

**The iniB, iniA and iniC GENES OF MYCOBACTERIA AND METHODS OF USE**

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**Background of the Invention**

This invention is based upon the discovery by the inventors of the iniB, iniA and iniC genes, and the proteins encoded by these genes which are induced by a broad class of antibiotics that act by inhibiting cell wall biosynthesis, 10 including the first line antituberculosis agents, isoniazid (INH) and ethambutol (EMB). The discovery of the iniB, iniA and iniC genes, and the proteins encoded by these genes will have important implications in the identification of drugs effective against *M. tuberculosis*, as well as the treatment of drug-resistant mycobacterial strains.

15 Many highly effective classes of antibiotics work by inhibiting microbial cell wall biosynthesis. In *M. tuberculosis* several antibiotics, including isoniazid and ethambutol, appear to act by this general mechanism.

EMB is a selective antimycobacterial drug recommended for clinical use in 1996 (Karlson, A.G., *Am Rev Resp Dis* 84, 905-906 (1961)). Today, EMB 20 remains an important component of tuberculosis treatment programs. Unfortunately, resistance to ethambutol has been described in 2-4% of clinical isolates of *M. tuberculosis* in the USA and other countries, and is prevalent among isolates from patients with multidrug-resistant tuberculosis (Bloch, AB., Cauthen, GM., Onorato, IM., et al. Nationwide survey of drug-resistant tuberculosis in the 25 United States. *JAMA* 271, 665-671 (1994)).

EMB targets the mycobacterial cell wall, a unique structure among prokaryotes which consists of an outer layer of mycolic acids covalently bound to peptidoglycan via the arabinogalactan (Besra, G.S. & Chatterjee, D. in *Tuberculosis. Pathogenesis, protection, and control* (ed Bloom, B.R.) 285-306 (ASM Press, 30 Washington DC, 1994)). Lipoarabinomannan, another cell wall component of

significant biological importance, shares with arabinogalactan the overall structure of the arabinan polymer (Chatterjee, D., et al., *J. Biol Chem* 266, 9652-9660 (1991)).

EMB inhibits the *in vivo* conversion of [<sup>14</sup>C]glucose into cell wall 5 arabinan (Takayama, K. & Kolburn, J.O., *Antimicrob Agents Chemother* 33, 143-1499 (1989)), and results in the accumulation of the lipid carrier decaprenyl-P- arabinose (Wolucka, B.A., et al., *J Biol Chem* 269, 23328-23335 (1994)), which suggest that the drug interferes with the transfer of arabinose to the cell wall acceptor. The synthesis of lipoarabinomannan is also inhibited in the presence of 10 EMB (Deng, L., et al. *Antimicrob Agents Chemother* 39, 694-701 (1995)), (Mikusova, K., et al., *Antimicrob Agents Chemother* 39, 2484-2489 (1995)); again, this indicates a specific effect on arabinan biosynthesis.

Isoniazid (INH) is a front-line drug in the treatment of tuberculosis. INH is a prodrug that requires activation by the catalase-peroxidase enzyme (katG) 15 to an active form (Zhang et al., (1992) *Nature* 358, 591-593). It is likely that INH acts by blocking mycolic acid biosynthesis as evidenced by the physical and biochemical changes that occur at the same time as INH toxicity (Winder and Collins, (1970) *J. Gen. Microbiol.* 63, 41; Davidson and Takayama, (1979) *Antibicrob. Agents Chemother.* 16, 104). Treatment with INH leads to the 20 accumulation of saturated hexacosanoic acid, and has been shown to inhibit the action of several enzymes thought to be involved in mycolic acid biosynthesis including InhA (Banerjee et al., (1994) *Science* 263, 227-230) and kasA (Mdluli et al., (1998) *Science* 280, 1607-1610).

Recent reports have documented a significant increase in the global 25 incidence of drug resistant tuberculosis. Thus, there is a need for the identification and characterization of new target genes and proteins to aid in screening for new drugs. This would require the identification of genes that participate in the biosynthesis of the mycobacterial cell wall and the identification of mutants of these

genes encoding proteins that confer resistance to drugs. While it is possible that the iniB, iniA, and iniC gene products are not in themselves targets for currently available antibiotics, these proteins may act to protect *M. tuberculosis* and other mycobacteria from toxic effects that occur when cell wall biosynthesis is inhibited 5 by antibiotics. Novel drugs that inhibit the iniB, iniA, and iniC proteins may therefore act synergistically with other cell wall active antibiotics and prove useful in treating tuberculosis, including drug resistant tuberculosis.

Current high throughput drug screens do not usually assay agents at high concentrations because nonspecific toxic effects are common. This strategy 10 may miss important compounds that could be modified to have higher potency. This is a particular concern for screening compounds against *M. tuberculosis* because many drugs may have difficulty penetrating through its lipid laden cell wall. Thus, there is a great need for new drug screens which overcome the deficiencies of the present screens for compounds against *M. tuberculosis*.

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#### Summary of the Invention

The present invention is directed to the nucleic acid sequences of the iniB, iniA and iniC genes, and the proteins encoded by these genes which are induced by a broad class of antibiotics that act by inhibiting cell wall biosynthesis, 20 including the first line antituberculosis agents, ethambutol (EMB) and isoniazid. The present invention further provides for the identification, isolation and characterization of these nucleic acid sequences and the proteins which they encode.

The present invention specifically provides purified and isolated 25 nucleic acid sequences of the iniB, iniA, and iniC genes, as well as mutated forms of these genes. The present invention also provides one or more single-stranded nucleic acid probes which specifically hybridize to the nucleic acid sequences of the iniB, iniA, and iniC genes, as well as mutated forms of these genes, and mixtures

thereof, which may be formulated in kits, and used in the detection of drug resistant mycobacterial strains.

The present invention also provides purified active iniB, iniA, and iniC proteins encoded by the iniB, iniA, and iniC genes. Also provided are 5 antibodies immunoreactive with the protein(s) expressed by the iniB, iniA, and iniC genes, and analogues thereof, as well as antibodies immunoreactive with the protein(s) expressed by the these genes.

Further provided by the present invention is a method of screening drugs or compounds to determine whether the drug or compound is effective 10 against *Mycobacterium tuberculosis*.

Additional objects of the invention will be apparent from the description which follows.

#### Brief Description of the Figures

15 **Figure 1:** Figure 1 shows induction of the iniA gene after treatment with different antibiotics. Autoradiographs of a Northern blot containing RNA from *M. tuberculosis* cultures treated either with no antibiotics; isoniazid 0.01  $\mu$ g/ml; isoniazid 0.1  $\mu$ g/ml; isoniazid 1  $\mu$ g/ml; ethambutol 5  $\mu$ g/ml; streptomycin 5  $\mu$ g/ml; and rifampin 5  $\mu$ g/ml. The blots were hybridized first with an iniA DNA probe 20 (top) to examine iniA induction; the blot was then stripped and re-hybridized with a 16S probe (bottom) to confirm equal RNA loading.

**Figures 2A and 2B:** Figures 2A and 2B show reverse transcription PCR of differentially expressed genes. Figure 2A: RNA was extracted from log phase *M. tuberculosis* strain Erdman either without (lanes 1-3) or with (4-6) isoniazid added 25 to the bacterial cultures for the last 18 hours. RNA from both cultures was equalized by comparison of the 16S band intensity. RT PCR using three ten-fold

dilutions of each RNA and either *iniA*, *asd* or 16S specific primers was performed. Induction of *iniA* and suppression of *asd* by isoniazid is demonstrated. The amount of 16S RT PCR product is similar for equivalent dilutions, indicating equal amounts of starting RNA. Lanes 7-8 are minus RT controls; and lane 9 a negative PCR control. Figure 2B: Lack of *iniA* induction in an isoniazid resistant strain. Cultures of isogenic BCG strain ATCC35735 which is susceptible to isoniazid (lanes 1-6), or ATCC35747 which is resistant to isoniazid (lanes 7-12), were incubated either in the presence or absence of isoniazid for the last 18 hours. Three ten-fold dilutions of RNA extracted from each culture were tested by RT PCR for *iniA* induction. Induction is seen only in the INH susceptible strain. Lanes 13-16 are minus RT controls; and lane 17 a negative PCR control containing no added template.

**Figure 3:** Figure 3 shows the results of the experiments directed to the induction of the *iniB* promoter.

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**Figure 4:** Figure 4 shows the results of the experiments directed to the induction of *iniB* by amino acids.

**Figure 5:** Figure 5 shows the results of the experiments directed to the induction of the *iniB* promoter as a function of growth phase.

**Figures 6A-6C:** Figures 6A-6C set forth the nucleic acid sequences of the *iniB*, *iniA* and *iniC* genes, and the promoter region of the *iniB* gene. MTCY279, genebank accession Z97991. Nucleotides 9048-9101, then nucleotides 1 - 159 of M. tuberculosis cosmid MTY13E10, genebank accession Z95324. For a total of 213 nucleotides. Nucleotide sequences of genes, numbering from MTY13E10 *iniB* 160-1559; *iniA* 1636-3558 and *iniC* 3555-5036. (Nucleotides 160-1559 of Seq ID No:2) (Nucleotides 3555-5036 of Seq ID No:2) (Nucleotides 1636-3558 of Seq ID No:2)

**Figure 7:** Figure 7 sets forth the amino acid sequences encoded by the iniB, (SEQ ID NO:4) (SEQ ID NO:5) iniA, and iniC genes.

#### Detailed Description of the Invention

5 The present invention is directed to the nucleic acid sequences of the iniB, iniA and iniC genes, and the proteins encoded by these genes which are induced by a broad class of antibiotics that act by inhibiting cell wall biosynthesis, including the first line antituberculosis agents, ethambutol (EMB) and isoniazid.

The present invention specifically provides purified and isolated nucleic acid  
10 sequences of the iniB, iniA, iniC and iniB promoter genes. Also provided are mutated forms of these nucleic acids. It is possible, that the iniB, iniA and iniC genes may form an operon, herein designated the "iniBAC operon" or the "iniA operon". As used herein, an "operon" is a cluster of related genes and their promoters that encode for open reading frames. The "iniBAC operon" as used  
15 herein consists of the iniB, iniA and iniC genes arranged in a single operon, as well as the sequences encoding the promoters for the iniBAC genes. The "wild type iniBAC operon" is herein defined as the normal form of the iniB, iniA, and iniC genes which express gene products, and includes degenerate forms. The "mutated iniBAC operon" is the mutated form of the normal iniBAC operon, which contains  
20 one or more deletions, insertions, point, substitution, nonsense, missense, polymorphism, or rearrangement mutations, or a combination thereof. As used herein, "nucleic acid" may be genomic DNA, cDNA or RNA, and may be the entire nucleic acid sequence comprising the iniB, iniA, and iniC genes, the nucleic acid sequence of the iniB gene and its promoter, the nucleic acid sequence of the iniB  
25 promoter, or any portion of the sequence thereof.

The present invention specifically provides for the iniB, iniA, and iniC nucleic acid sequences isolated from *M. tuberculosis*. These sequences are set forth in Figure 6. The present invention also provides for the iniB, iniA, and iniC nucleic

acid sequences which encodes the amino acid sequence set forth in Figure 7. The present invention provides for the nucleic acid sequence comprising the iniB promoter region set forth in Figure 6. Figure 6 indicates the position of the iniB promoter, however, it is to be understood that the iniB promoter may consist of 5 additional nucleotides upstream from the iniB promoter region indicated in Figure 6.

The present invention further provides for mutated nucleic acid sequences of the iniB, iniA, and iniC nucleic acid sequences. These mutation(s) may be 10 deletions, insertions, substitutions, missense, nonsense, point or rearrangement mutations, or a combination thereof.

The nucleic acid sequences of the iniB, iniA, and iniC genes can be prepared several ways. For example, they can be prepared by isolating the nucleic acid sequences from a natural source, or by synthesis using recombinant DNA techniques. In addition, mutated nucleic acid sequences of the iniB, iniA, and iniC 15 genes can be prepared using site mutagenesis techniques. The amino acid sequences may also be synthesized by methods commonly known to one skilled in the art (*Modern Techniques of Peptide and Amino Acid Analysis*, John Wiley & Sons (1981); M. Bodansky, *Principles of Peptide Synthesis*, Springer Verlag (1984)). Examples of methods that may be employed in the synthesis of the amino acids 20 sequences, and mutants of these sequences include, but are not limited to, solid phase peptide synthesis, solution method peptide synthesis, and synthesis using any of the commercially available peptide synthesizers. The amino acid sequences, and mutants thereof, may contain coupling agents and protecting groups used in the synthesis of the protein sequences, and are well known to one of skill in the art.

25 The present invention also provides single-stranded nucleic acid probes and mixtures thereof for use in detecting drug resistance caused by a mutated nucleic acid of the iniB, iniA, or iniC genes. The nucleic acid probes may be DNA, cDNA, or RNA, and may be prepared from the mutated and/or wild type nucleic acid

sequences comprising the iniB, iniA, or iniC genes. The probes may be the full length sequence of the nucleic acid sequences comprising the iniB, iniA, or iniC genes, or fragments thereof. Typical probes are 12 to 40 nucleotides in length.

5 The probes may be synthesized using an oligonucleotide synthesizer, and may be labeled with a detectable marker such as a fluorescence, enzyme or radiolabeled markers including  $^{32}\text{P}$  and biotin, and the like. Combinations of two or more labeled probes corresponding to different regions of the iniB, iniA, or iniC genes also may be included in kits to allow for the detection and/or analysis of the iniB, iniA, and iniC genes by hybridization.

10 Specifically, the nucleic acid sequences of the iniB, iniA, or iniC genes may be used to produce probes which can be used in the identification, treatment and prevention of diseases caused by microorganisms and to determine whether various drugs are effective against mycobacterial strains.

The present invention also provides purified active iniB, iniA, and iniC proteins, encoded by the iniB, iniA, and iniC genes. The proteins may be expressed by the wild type or mutated nucleic acid sequences of the iniB, iniA, and iniC genes, or an analogue thereof. As used herein, "analogue" means functional variants of the wild type protein, and includes iniB, iniA, and iniC proteins isolated from bacterial sources other than mycobacteria, as well as functional variants thereof. The 20 proteins may also be isolated from native cells, or recombinantly produced.

The present invention also provides antibodies immunoreactive with the proteins expressed by the iniB, iniA, and iniC genes, and analogues thereof, as well as antibodies immunoreactive with the proteins expressed by the mutated nucleic acid sequences of the iniB, iniA, and iniC genes. The antibodies may be polyclonal or monoclonal and are produced by standard techniques. The antibodies may be labeled with standard detectable markers (e.g. chemiluminescent detection systems and radioactive labels such as  $^{125}\text{I}$ ) for detecting the wild type and mutated iniB, iniA, and iniC genes. The antibodies may also be presented in kits with detectable

labels and other reagents and buffers for such detection.

The present invention also provides for a method of assessing the susceptibility of a mycobacterium to EMB and/or isoniazid in a clinical sample comprising isolating the mycobacterial chromosomal DNA from a clinical sample, 5 preparing oligonucleotides utilizing the wild-type or mutant iniB, iniA, or iniC nucleic acid sequences, amplifying the region of the iniB, iniA, or iniC gene from the clinical sample, and determining whether a mutated iniB, iniA, or iniC gene exists in the mycobacterial strain in the clinical sample.

The mycobacteria that may be assessed by this method of the present 10 invention include, but are not limited to, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium smegmatis*, *Mycobacterium bovis BCG*, *Mycobacterium leprae*, *Mycobacterium africanum*, and *Mycobacterium intracellulare*.

Non-limiting examples of clinical samples that may be assessed by the methods 15 of the present invention are urine, feces, blood, serum, mucus, cerebrospinal fluid, and any mixture thereof.

The present invention also provides for a method of treating a mycobacterial infection in a subject by obtaining anti-DNA or anti-RNA nucleic acid sequences capable of inhibiting the mRNA activity of the iniB, iniA, or iniC genes of a mycobacterium, utilizing a wild type or the mutant nucleic acid of the iniB, iniA, 20 or iniC genes, and administering an amount of said nucleic acid sequences, either alone or in combination with other compositions to treat the mycobacterial infection in a subject.

The anti-DNA or anti-RNA nucleic acid sequences employed in the method may be mutant or wild-type nucleic acid sequences of the iniB, iniA, or iniC genes. 25 The mutant nucleic acid sequence may contain one or more deletions, insertions, substitutions, missense, nonsense, polymorphisms, point, or rearrangement mutations. The mutant nucleic acid sequence may be single-stranded, and labeled with a detectable marker.

Non-limiting examples of infections that can be treated using the methods of the present invention include those caused by mycobacteria selected from the group consisting of *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium smegmatis*, *Mycobacterium bovis BCG*, *Mycobacterium leprae*,  
5 *Mycobacterium africanum*, and *Mycobacterium intracellulare*.

The nucleic acid sequences of the present invention are administered in conjunction with a suitable pharmaceutical carrier. Representative examples of suitable carriers include, but are not limited to, mineral oil, alum, and synthetic polymers. Vehicles for vaccines are well known in the art and the selection of a  
10 suitable vehicle is deemed to be within the scope of those skilled in the art from the teachings contained herein. The selection of a suitable vehicle is also dependent on the manner in which the nucleic acid sequences are to be administered. The nucleic acid sequences may be administered orally, enterally, subcutaneously, intraperitoneally, intravenously, or intranasally. Accordingly, as used herein,  
15 "subject" may be an embryo, fetus, newborn, infant, or adult. Further, as used herein "treating" is contacting a mycobacterium with the nucleic acids of the present invention, alone or in combination with other compositions.

The present invention additionally provides for the use of the nucleic acid sequences of the iniB, iniA, or iniC genes of the present invention as vaccines, or to  
20 improve existing vaccines.

Non-limiting examples of mycobacterial infections that can be treated using the vaccines of the present invention include those caused by mycobacteria selected from the group consisting of *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium smegmatis*, *Mycobacterium bovis BCG*, *Mycobacterium leprae*,  
25 *Mycobacterium africanum*, and *Mycobacterium intracellulare*. For example, *M. tuberculosis* complex strains that have mutations in the iniB, iniA or iniC genes might have reduced virulence. In addition, mutated genes of *M. tuberculosis* and

M. bovis can be added to BCG or tuberculosis vaccines to provide attenuated mutant tuberculosis vaccines. These vaccines can be used to treat and prevent a wide variety of diseases, including tuberculosis, human immunodeficiency viral infection, polio, leprosy, malaria, tetanus, diphtheria, influenza, measles, mumps, 5 hepatitis and rabies.

The present invention also provides for novel vector constructs and a novel methods of using the constructs for screening drugs or compounds to determine whether the drug or compound is effective against *Mycobacterium tuberculosis*.

Specifically provided by the present invention are vector constructs 10 comprising a DNA sequence comprising the iniB promoter region. The DNA encoding the iniB promoter region may be obtained several ways. For example, it can be prepared by isolating the iniB promoter region DNA sequences from a natural source, by synthesis using recombinant DNA techniques, by synthesis using a DNA synthesizer, or by amplification using the polymerase chain reaction. Such 15 vectors may be constructed by inserting the DNA sequence comprising the iniB promoter region into a suitable vector. The term "inserted" as used herein means the ligation of a foreign DNA fragment and vector DNA by techniques such as the annealing of compatible cohesive ends generated by restriction endonuclease digestion or by use of blunt end ligation techniques. Other methods of ligating DNA 20 molecules will be apparent to one skilled in the art.

Vectors suitable for expression of a DNA sequence comprising the iniB promoter region in a cell are well known to those skilled in the art and include pQE-8 (Qiagen), pET-3d (Novagen), pProEx-1 (Life Technologies), pFastBac 1 (Life Technologies), pSFV (Life Technologies), pcDNA II (Invitrogen), pSL301 25 (Invitrogen), pSE280 (Invitrogen), pSE380 (Invitrogen), pSE420 (Invitrogen), pTrcHis A,B,C (Invitrogen), pRSET A,B,C (Invitrogen), pYES2 (Invitrogen), pAC360 (Invitrogen), pVL1392 and pVL1392 (Invitrogen), pCDM8 (Invitrogen), pcDNA I

(Invitrogen), pcDNA I(amp) (Invitrogen), pZeoSV (Invitrogen), pcDNA 3 (Invitrogen), pRc/CMV (Invitrogen), pRc/RSV (Invitrogen), pREP4 (Invitrogen), pREP7 (Invitrogen), pREP8 (Invitrogen), pREP9 (Invitrogen), pREP10 (Invitrogen), pCEP4 (Invitrogen), pEBVHis (Invitrogen),  $\lambda$ Pop6, pBR322, pUC18, pUC19, pHHSV-  
5 106, pJS97, pJS98, M13mp18, M13mp19, pSPORT 1, pGem, pSPORT 2, pSV  
SPORT 1, pBluescript II,  $\lambda$ ZapII,  $\lambda$ gt10,  $\lambda$ gt11,  $\lambda$ gt22A, and  $\lambda$ ZIPLOX. Other vectors  
would be apparent to one skilled in the art.

The vector constructs of the present invention contain a nucleotide sequence  
encoding suitable regulatory elements so as to effect expression of the vector  
10 construct in a suitable host cell. Those skilled in the art will appreciate that a  
variety of ~~enchanters~~<sup>enhancers</sup> promoters, and genes are suitable for use in the constructs  
of the invention, and that the constructs will contain the necessary start,  
termination, ribosomal binding sequences, and control sequences for proper  
transcription and processing of the iniB promoter region when the vector construct  
15 is introduced into a host cell.

The vector constructs may contain one or more reporter genes. Examples of  
reporter genes that may be employed include, but are not limited to, luciferase from  
Vibrio or of firefly origin; green fluorescent protein; beta-galactosidase; beta-  
glucuronidase; or catechol dehydrogenase and a strong mycobacterial promoter  
20 which controls expression of the reporter molecule-encoding gene. The reporter  
gene may be part of an existing vector, or it may be inserted during the course of  
the construction of the vector.

The vector constructs may also contain one or more expressible and  
selectable genes of interest. Examples of selectable markers that may be employed  
25 include, but are not limited to, leucine C, leucine D, chloramphenicol resistance  
gene, tetracycline resistance gene, hygromycin resistance gene, gentamycin  
resistance gene, B-galactosidase gene, ampicillin resistance gene, herpes simplex  
virus gene, vaccine virus thymidine kinase gene, adenine phosphoribosyltransferase

gene, hypoxanthine-guanine phosphoribosyltransferase gene, aspartate transcarbamylase gene, ornithine decarboxylase gene, aminoglycoside phosphotransferase gene, hygromycin-B-phosphotransferase gene, xanthine-guanine phosphoribosyltransferase gene, tryptophan synthetase gene, histidinol dehydrogenase gene, multiple drug resistance gene, dihydrofolate reductase gene, CAD (carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotate) gene, adenosine deaminase gene, asparagine synthetase gene, and glutamine synthetase gene. The selectable marker may be part of an existing vector, or it may be inserted during the course of the construction of the vector.

10 The present invention further provides a cell transformed with the novel vector constructs of the present invention. The cell may be eukaryotic or prokaryotic. Suitable host cells include, but are not limited to, bacterial cells such as *E. coli*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Bacillus megaterium*, eukaryotic cells such as *Pichia pastoris*, *Chlamydomonas reinhardtii*, *Cryptococcus neoformans*, *Neurospora crassa*, *Podospora anserina*, *Saccharomyces cerevisiae*, *Saccharomyces pombe*, *Uncinula necator*, cultured insect cells, cultured chicken fibroblasts, cultured hamster cells, cultured human cells such as HT1080, MCF7, 143B and cultured mouse cells such as EL4 and NIH3T3 cells.

15 In a preferred embodiment of the invention, the cell transformed with the vector construct of the present invention is a mycobacterium. Non-limiting examples of mycobacterium which may be transformed with the vector construct of the present invention are *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium smegmatis*, *Mycobacterium bovis BCG*, *Mycobacterium leprae*, *Mycobacterium africanum*, and *Mycobacterium intracellulare*.

20 The vector constructs of the present invention can exist in integrated or unintegrated form within the host cell and are capable of autonomous replication

when in unintegrated form. The term "host cell" as used herein means the bacterial or eukaryotic cell into which the vector is introduced. As used herein, "introduced" is a general term indicating that one of a variety of means has been used to allow the vector to enter the intracellular environment of the host cell in such a way that 5 it exists in stable form therein.

The constructs may be introduced into host cells by a variety of gene transfer methods known to those skilled in the art, such as electroporation, treatment with calcium chloride, DEAE dextran, cationic liposome fusion, protoplast fusion, DNA coated-microprojectile bombardment, and infection with recombinant replication-10 defective retroviruses. Other techniques will be obvious to one skilled in the art. The term "transformation" will be used herein as a general term to denote the introduction of vector into a bacterial or eukaryotic host cell. As such, it encompasses transformation of bacterial cells and transfection, transduction and related methods in eukaryotic cells.

15 The present invention also provides for the use of the vector constructs containing a DNA sequence comprising the iniB promoter region for screening drugs or compounds to determine whether the drug or compound is effective against *Mycobacterium tuberculosis*. This method comprises transforming the vector construct into a mycobacterium. Non-limiting examples of mycobacteria which 20 may be used in this method include *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium smegmatis*, *Mycobacterium bovis BCG*, *Mycobacterium leprae*, *Mycobacterium africanum*, and *Mycobacterium intracellulare*. The mycobacterium is cultured, preferably to an OD of 0.2-0.8. The drug or compound to be tested is then added to the culture and the mycobacteria are allowed to grow further. After 25 a determined period of time, the culture is measured for induction of the iniB promoter. Induction is preferably determined by the expression of a reporter gene, such as lacZ or luciferase. Induction of the iniB promoter is a positive indication of the effectiveness of the drug or compound against the *Mycobacterium tuberculosis*

cell wall and any other mechanism to be determined.

The present invention is described in the following Experimental Details Section, which is set forth to aid in an understanding of the invention, and should not be construed to limit in any way the invention as defined in the claims which 5 follow thereafter.

**Experimental Details Section**

**Isolation and identification of the iniB, iniA, and iniC genes.**

**Libraries and plasmids.** Cosmid libraries were constructed by ligation of 10 *Sau*3A partial digests of *M. tuberculosis* H37Rv into pYUB328 (14). Plasmid libraries were constructed by ligation of complete *Pst*I or *Sac*I digests of *M. tuberculosis* H37Rv into pUC19 (15).

**Creation of ribosomal free customized amplification libraries.** One thousand cosmid library clones were inoculated into "master" 96 well microtiter 15 plates containing L broth and ampicillin 50  $\mu$ g/ml, transferred by a pronged "frog" onto Biotrans nylon membranes (ICN Pharmaceuticals, Costa Mesa, CA), and hybridized separately with [ $\alpha^{32}$ P] radiolabeled (Megaprime labeling kit, Amersham, Arlington Heights, IL) PCR probes to *M. tuberculosis* ribosomal 5S, 16S, and 23S genes. Fourteen cosmids containing ribosomal DNA were identified; non- 20 ribosomal cosmids were re-inoculated from master plates and individually cultured. Cosmids were extracted by SDS/alkaline lysis (17) in pools of 16. Cosmid DNA was pooled, digested with *Pac*I, which does not restrict the *M. tuberculosis* genome, and insert DNA was purified from an agarose gel by electro-elution. Approximately 1  $\mu$ g of precipitated DNA was digested with *Alu*I and 100 ng run on a 2% NuSieve 25 GTG low melting point agarose gel (FMC Bioproducts, Rockland, ME). Marker DNA was run simultaneously in a separate gel to avoid cross contamination of samples. The gels were aligned, and the section corresponding to 400 - 1,500 base pairs of the *Alu*I digest was excised. Five  $\mu$ l of gel slice was ligated with 1  $\mu$ l of

Uniamp *Xba*I adapters 2 pmol/ $\mu$ l (Clonetech, Palo Alto, CA) in 20  $\mu$ l total volume. Ten  $\mu$ l of the ligation was PCR amplified with 2  $\mu$ l of 10  $\mu$ M Uniamp primers (Clonetech), 1X vent polymerase buffer and 0.8 units of Vent (exo-) polymerase (New England Biolabs, Beverly, MA) in 100  $\mu$ l total volume. After a five minute hot 5 start, ten cycles of PCR with one minute segments of 95 $^{\circ}$ C, 65 $^{\circ}$ C, and 72 $^{\circ}$ C, were followed by the addition of 3.2 units of Vent (exo-) polymerase and 27 additional cycles of 95 $^{\circ}$ C for one minute, 65 $^{\circ}$ C for two minutes, and 72 $^{\circ}$ C for three minutes. Uniamp primer sequence: 5'-CCTCTGAAGGTTCCAGAATCGATAG-3'; Uniamp *Xba*I (SEQ ID NO:6) adapter sequence top strand: 5'-CCTCTGAAGGTTCCAGAATCGATAGCTCGAGT-3'; 10 bottom strand: 5'-P-ACTCGAGCTATCGATTCTGGAACCTTCAGAGGTTT-3'. (SEQ ID NO:7) (SEQ ID NO:8)

**RNA extraction.** Mycobacterial cultures were grown to mid log phase in Middlebrook 7H9 media supplemented with OADC, 0.05% Tween 80, and cyclohexamide (18) (for some experiments antibiotics were added for the last 18 hours), pelleted, resuspended in chloroform/methanol 3:1, and vortexed for 60 15 seconds or until the formation of an interface. RNA was extracted with five volumes of Triazole (Life Technologies, Gaithersburg, MD), the aqueous layer precipitated in isopropanol, redissolved in 4M GTC and extracted a second time with Triazole.

**Positive selection and generation of PCR probes.** One  $\mu$ g of RNA was 20 reverse transcribed with 7.7  $\mu$ g biotin labeled random hexamers and biotin dATP (one tenth total dATP) using superscript II (Gibco BRL, Grand Island, NY) at 50 $^{\circ}$ C for one hour, RNase H was then added for one half hour at 37 $^{\circ}$ C. Three hundred ng of CAL, 20  $\mu$ g of salmon sperm DNA, and 20  $\mu$ g of tRNA were added to the cDNA for a final volume of 150  $\mu$ l. The sample was phenol/chloroform extracted 25 twice, ethanol precipitated overnight, resuspended in 6  $\mu$ l of 30 mM EPPS (Sigma), pH 8.0/ 3 mM EDTA, overlain with oil, and heated to 99 $^{\circ}$ C for 5 minutes, then 1.5  $\mu$ l of 5 M NaCl preheated to 69 $^{\circ}$ C was quickly added (19). The sample was incubated at 69 $^{\circ}$ C for three to four days, then diluted with 150  $\mu$ l of incubation

buffer (1X TE, 1 M NaCl, 0.5% Tween 20) that had been preheated to 69°C, and 50  $\mu$ l of washed, preheated streptavidin coated magnetic beads (Dynal, Oslo, Norway) were then added. The sample was then incubated at 55°C with occasional mixing for 30 minutes, washed three times at room temperature and three times 30 5 minutes at 69°C with 0.1% SDS, 0.2X SSC by placing the microfuge tubes into a larger hybridization tube in a rotating microhybridization oven (Bellco, Vineland, N.J.). The sample was then washed with 2.5 mM EDTA and eluted by boiling in 80  $\mu$ l of water. PCR was performed as in the CAL preparation using 20  $\mu$ l of sample. The product of this PCR reaction is termed "PCR probes".

10 **Colony array hybridizations.** Genomic plasmid library arrays were prepared by Genome Systems (St. Louis, MO) by robotically double spotting 9,216 colonies from the *Pst*I and *Sac*I plasmid libraries onto replicate nylon membranes. PCR probes were labeled by random priming with  $[\alpha^{32}P]$  dCTP (Megaprime labeling kit, Amersham) for at least 6 hours, hybridized to the colony arrays in 15 Rapid-hyb buffer (Amersham), washed at 69°C in 0.1X S.C., 0.1% SDS, and visualized by autoradiography. Double spotted colonies which hybridized at different intensities with two PCR probes were selected for further analysis.

20 **Northern blots.** Five  $\mu$ g of each RNA sample were analyzed by northern blot with Northern Max kits (Ambion, Austin, TX) in a 1% denaturing agarose gel, probed with inserts of differentially expressed plasmids labeled by random priming with  $[\alpha^{32}P]$  dCTP, and visualized by autoradiography.

25 **Southern blots.** Plasmid or genomic DNA was digested with restriction enzymes, subjected to electrophoresis in a 1% agarose gel and transferred by capillary action to Biotrans nylon membranes. The blots were hybridized and washed as in "colony array hybridizations" above, and visualized by autoradiography.

**Reverse Transcription PCR.** (See also Figure 2) One microgram of RNA was reverse transcribed using the appropriate reverse PCR primer and superscript II at

50°C. For *iniA* and *asd*, three serial ten-fold dilutions of cDNA were made; 16S cDNA was diluted 1 in 10<sup>6</sup>, 1 in 10<sup>7</sup>, and 1 in 10<sup>8</sup>. PCR was performed with Taq polymerase and 1X PCR buffer (Gibco BRL) containing 2 mM MgCl<sub>2</sub> for 25 cycles annealing at 60°C for *iniA*; 35 cycles annealing at 58°C for *asd*; 25 cycles annealing at 63°C for 16S. PCR products were analyzed on a 1.7% agarose gel, images were stored to disk by digital camera (Appligene, Pleasanton, CA), and the amounts of PCR product were calculated by densitometry (Imaging Software, National Institute of Health, Bethesda, MD). Primers used for *iniA*:  
5'-GCGCTGGCGGGAGATCGTCAATG-3' (SEQ ID NO: 9)  
10 for *asd*: 5'-TCCCGCCGCCAACACCTA-3' (SEQ ID NO: 10), 5'-GGATCCGGCCGACCAAGAGA-3' (SEQ ID NO: 11); for 16S: 5' - G G A G T A C G G C C G C A A G G C T A A A A C - 3' (SEQ ID NO: 12), 5'-CAGACCCCCGATCCGAACTGAGACC-3' (SEQ ID NO: 13).

**Induction of the *iniB* promoter.** The 213 base pair *iniB* promoter region was cloned into a *lacZ* and fflux reporter construct and transformed into BCG. Cells were cultured to an OD590 of 0.2-0.8 and then split into different aliquots. Individual aliquots of cells were treated with antibiotics, or other agents and cultured for an additional 24 and 48 hours.  $\beta$ -galactosidase activity of the culture was measured by an O-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) assay at various time points. Luciferase activity was measured by adding luciferase to the cultures and measuring relative light units in a luminometer. Induction was calculated as the  $\beta$ -galactosidase or luciferase activity of antibiotic treated cells over  $\beta$ -galactosidase or luciferase activity of untreated control cultures at the same time point.

**Induction of the *iniB* promoter by amino acids that block cell wall synthesis.** D-threonine, but not L-threonine inhibits cell wall biosynthesis by disrupting D-ala/D-ala cross-linking of the peptidoglycan cell wall. BCG containing the *iniB/lacZ* construct were treated with various antibiotics and amino acids.

Induction of the *iniB* promoter at 24 hours with D-threonine is comparable to that of isoniazid and Unisyn (amoxicillin/sulbactam). Modest induction is also seen with 1% glycine which is also known to weaken the mycobacterial cell wall. However, the L-threonine control did not cause induction.

5       **Induction of the *iniB* promoter as a function of growth phase.** One BCG culture containing the *iniA/lacZ* construct was diluted in media to an OD<sub>590</sub> of less than 0.1. The culture was placed at 37°C with shaking. Two aliquots were removed from the culture at 24 hour intervals. Ethambutol was added to one aliquot; the second aliquot was used as a no antibiotics control.  $\beta$ -galactosidase activity was measured for both aliquots 24 hours later by ONPG assay and the process was repeated.  $\beta$ -galactosidase activity is shown for the un-treated control (squares), or ethambutol treated aliquot (diamonds) as a function of the OD<sub>590</sub> of the culture when the aliquots were removed (columns).

10      **Use of the *iniB* promoter to screen compounds for new cell wall active drugs.** Mycobacteria, preferably *M. tuberculosis* but also other mycobacteria are transformed with a reporter construct under the control of the *iniA* promoter sequence as set forth in Figure 6 or a smaller portion of this sequence, or a larger sequence. These transformed strains would be used to screen for compounds with cell wall activity either in liquid or solid phase assays. For testing using liquid 15 phase assays, the transformed strains would be cultured to an OD of approximately 0.2 to 0.8 and then placed into microwell plates or other multiwell or multitube containers. Compounds to be tested would be added to each well, and the samples would be cultured for an additional period of time, usually between five and 48 hours. The growing strains could also be added to wells that already contained the 20 compounds to be tested. These well would be cultured for similar periods of time. The wells would then be assayed for activity of the reporter molecule preferably luciferase or beta galactosidase. Compounds that caused significant induction of 25 the *iniB* promoter would be identified by comparing the reporter activity in the

wells containing the compounds to control wells to which either no compounds had been added, or to which suitable control compounds had been added. Significant induction would be any increase of the reporter in wells containing the test compounds over reporter activity in the control wells. Preferably the induction 5 would be greater than two fold, more preferably it would be greater than five fold, and even more preferably it would be greater than tenfold. However, it might be determined that low levels of induction could indicate compounds with the potential to be modified for increased activity.

**For solid phase assays.** The transformed strains are grown in agar or top 10 agar and then compounds to be tested would be added on top of the growing strains. Compounds could be added to the agar using many methods, for example the compounds could be contained in small particles that would be dropped onto the agar in defined arrays. Induction of the promoter would be identified by a color change or other change in the media surrounding the compound, for example 15 due to beta galactosidase activity.

### **B. Results**

**Detection and evaluation of differential gene expression.** Differentially expressed genes were determined by examining the differential hybridization 20 patterns of the PCR probes referred to in Materials and Methods: Positive selection and generation of PCR probes. PCR probes derived from INH- and INH+ RNA samples were radiolabeled and hybridized to replica membranes containing arrays of colonies from an *M. tuberculosis* genomic library. Hybridization signals to most colonies were equal when small differences in background were accounted for, but 25 a subset of colonies was found to hybridize more strongly to either the INH- or INH+ probe. Six colonies were selected for further evaluation; five hybridized more strongly with the INH+ probe (P1-P5) and one hybridized more strongly with the INH- probe (P6). Differential hybridization was confirmed for P1, P2, P3, and

P6 by re-hybridizing the INH- and INH+ PCR probes to duplicate Southern blots of the excised plasmid inserts. P1 and P6 hybridized almost exclusively to the appropriate of the PCR probes, while P2 and P3 hybridized to both probes but with different intensities. P4 and P5 were found not to hybridize differentially on 5 secondary screen. The ends of the plasmid inserts were sequenced and aligned to the completely sequenced *M. tuberculosis* genome (20). P1 and P2, which encoded sequences that hybridized almost exclusively with the INH+ probe were homologous to a set of predicted proteins. P1 encoded a sequence identical to Rv0342, a large open reading frame that appeared to be the second gene of a 10 probable three gene operon. This open reading frame was named *iniA* (isoniazid induced gene A), and the upstream open reading frame Rv0341, was named *iniB*. P2 encoded a sequence that was not complementary to P1, but that was identical to the third gene in the same probable operon Rv0343, this open reading frame was named *iniC*. A putative protein encoded by the *iniA* gene was found to contain a 15 phosphopantetheine attachment site motif (21) suggesting that it functions as an acyl carrier protein. Both *iniA* and *iniC* lacked significant homology to other known genes but were 34% identical to each other. A sequence similarity search demonstrated that *iniB* had weak homology to alanine-glycine rich cell wall structural proteins (22). Northern blot analysis using excised inserts to probe total 20 RNA from *M. tuberculosis* cultured in the presence or absence or different antibiotics verified that *iniA* was strongly induced by isoniazid and ethambutol, drugs that act by inhibiting cell wall biosynthesis but not by rifampin or streptomycin, agents that do not act on the cell wall (Figure 1). P3, which also encoded a sequence that preferentially hybridized to the INH+ probe contained a 5 kb insert spanning *M. 25 tuberculosis* cosmids MTCYH10 and MTCY21D4. This region contained multiple small open reading frames, most with no known function. Northern blot analysis using the 5 kb insert as a probe confirmed that P3 preferentially hybridized to RNA

from *M. tuberculosis* that had been cultured in the presence of isoniazid (data not shown). P6, which encoded a sequence hybridizing predominantly with the INH-probe was found to encode L-aspartic- $\beta$ -semialdehyde dehydrogenase (*asd*). The *asd* gene is an important component of the diaminopimelate pathway required for biosynthesis of the peptidoglycan component of bacterial cell walls. Modulation of *asd* by a cell wall antibiotic such as isoniazid is not unexpected.

Reverse transcription (RT) PCR assays confirmed differential gene expression of both *asd* and *iniA* (Figure 2A), as well as of *iniB* and *iniC* (data not shown). As predicted, *iniA* was strongly induced by isoniazid (70 fold induction by densitometry), while *asd* was repressed (17 fold). Induction of *iniA* was also tested in two isogenic strains of BCG that were either sensitive or resistant to isoniazid. The resistant phenotype was conferred by a mutation in *katG* which normally converts isoniazid from a prodrug to its active form (23). Induction of *iniA* was seen only in the susceptible BCG strain demonstrating the requirement for isoniazid activation.

### C. Discussion

A three gene operon (the *iniA* operon) was discovered in *M. tuberculosis* that was strongly induced by both isoniazid and ethambutol. A 213 base pair sequence containing the *iniB* promoter was cloned into a *lacZ* reporter construct. Using this construct, it is herein demonstrated that the *iniB* promoter is induced by a wide range of cell wall active compounds but not by antibiotics or other stresses that do not act on the cell wall (Figure 3 and Figure 5). The *iniB* promoter is induced by antibiotics that act on very different targets within the cell wall including isoniazid which inhibits mycolic acid biosynthesis, EMB which inhibits arabinan and lipoarabinomannan biosynthesis, cycloserine which inhibits peptidoglycan cross linking and amoxicillin/sulbactam which inhibits penicillin binding proteins. The

iniA gene is also induced by D-threonine, an amino acid that substitutes for D-alanine and inhibits peptidoglycan biosynthesis. In contrast, L-threonine has a minimal effect on iniA transcription (Figure 4). The induction is not an artifact of cell wall breakdown and increased release of the  $\beta$ -galactosidase reporter because

5 iniB promoter induction can be reversed by co-administration of the RNA polymerase inhibitor rifampin (Figure 3). Induction has been demonstrated only during log phase growth of the recombinant BCG strain containing the reporter construct (Figure 5). This may be due to the intrinsic property of the promoter but may also reflect the mechanisms of action of the antibiotics available for testing.

10 It is possible that the iniA promoter is also inducible in stationary phase. This hypothesis would need to be tested with a compound that was able to disrupt cell wall biosynthesis during the stationary phase of the cell cycle.

The iniB promoter may be used in a reporter construct to rapidly screen compounds for new cell wall active drugs. Screening for iniB promoter induction

15 would also permit drugs to be assayed at higher than normal concentrations because it will be possible to distinguish between cell wall activity and nonspecific effects on cell growth. If the iniB promoter is inducible during stationary phase, then this strategy could be used to discover drugs that could be effective on latent or persistent infections.

20 While the foregoing invention has been described in detail for purpose of clarity and understanding, it will be appreciated by one skilled in the art from a reading of the disclosure that various changes in form and detail can be made without departing from the true scope of the invention in the appended claims.

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